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(54) Title: ASSAYS AND NOVEL CELLULAR TARGETS FOR THERAPEUTIC AGENTS TO TREAT RETROVIRAL INFECTION		
(57) Abstract Assays and test kits for identifying compounds that can interfere with early steps of the retroviral life cycle in a host cell are provided. Assays and test kits for identifying novel cellular enzymes needed to complete integration of retroviral DNA into cellular DNA are also provided. These assays are based on the discovery that cellular enzymes are needed to complete the repair steps of retroviral integration, and lack of such enzyme(s) causes cell death upon infection with a retrovirus. One required enzyme is a DNA-dependent protein kinase (DNA-PK). Methods for interfering with the retroviral life cycle in an infected host cell are also provided, which involve inhibiting DNA-PK or any other enzyme needed to complete the repair step of retroviral integration.		

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**ASSAYS AND NOVEL CELLULAR TARGETS FOR
THERAPEUTIC AGENTS TO TREAT RETROVIRAL INFECTION**

This application claims priority to U.S. Provisional Application No. 60/101,172, filed September 21, 1998, the entirety of which is incorporated by reference herein.

5 This invention was made with United States government support awarded by the NIH-NCI, Grant No. CA71515. The United States government has certain rights in this invention.

10 **FIELD OF THE INVENTION**

 This invention relates to the field of retroviruses and pathological conditions caused by retroviruses. In particular, this invention provides new assays to screen for anti-retroviral drugs, and a novel
15 cellular target for drug development.

BACKGROUND OF THE INVENTION

 Several scientific publications are referenced in this patent application to describe the state of the
20 art to which the invention pertains. Each of these references is incorporated by reference herein, in its entirety.

 Retroviruses cause several diseases and pathological conditions, including a variety of tumors
25 and leukemias. For instance, human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) in humans. Another significant disorder, adult T-cell leukemia-lymphoma, is

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caused by the retrovirus HTLV I (human t-cell leukemia virus type I). HTLV I also has been associated with other diseases, such as tropical spastic paraparesis and HTLV I-associated myelopathy. Moreover, many animal
5 diseases of agricultural and veterinary importance are known to be caused by retroviruses. These include avian sarcoma leukosis virus (ASLV), feline leukemia virus (FeLV), bovine immunodeficiency virus (BIV) and equine infectious anemia virus (EIAV), among others.

10 The development of antiviral agents to reduce or eliminate retrovirus-caused diseases has been a focus of intensive research over the past several years. One major difficulty in this effort has been the propensity of the viruses to mutate in response to exposure to a
15 particular antiviral agent and for resistant mutants to be selected. It is for this reason that "cocktails" comprising two or more antiviral agents often provide more effective treatment for retroviral infection than do single agents. However, over time, even a cocktail can
20 become ineffective due to the virus's ability to develop resistance to the antiviral agents.

Retroviruses encode several enzymes that are assembled into the virus particle and whose activities catalyze essential steps in the infectious cycle: (a)
25 protease (PR), (b) the polymerase and (c) ribonuclease H (RNase H) activities of reverse transcriptase (RT), and (d) integrase (IN). Following attachment and penetration of the retrovirus into the host cell, the RT activities catalyze reverse transcription of the viral RNA into DNA,
30 and IN catalyzes the integration of the retroviral DNA into the host cell DNA. The cellular transcription and translation machinery is used to produce viral RNA from the integrated viral DNA and thereafter to produce the viral proteins.

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Retroviral integration proceeds in three distinct steps, the first two of which are catalyzed by IN, and require specific sequences at the ends of the viral DNA, including a conserved CA dinucleotide. In the first step, referred to as "processing," two nucleotides (typically) are removed from the 3' ends of the viral DNA, immediately adjacent to the CA dinucleotide. In the second step, referred to as "joining," these new 3'-OH ends are joined to staggered phosphorus atoms in the host DNA. The resultant molecule comprises the viral DNA inserted into the host DNA with gaps on both strands due to the staggered nature of the joining reaction. Hence, the third step of integration, referred to as "repair," comprises filling in the gaps in host DNA produced by the staggered insertion (catalyzed by a polymerase activity), removing the non-complementary overhangs at the 5' ends of the viral DNA (catalyzed by a nuclease activity), and completion of the covalent closure (catalyzed by a ligase activity).

In *in vitro* studies, IN has been shown to catalyze the processing and joining steps of integration, but not the repair steps. It has been surmised that cellular enzymes may be required (either with or without the participation of IN) for these steps; however, the identity of the enzyme(s) involved has not been elucidated. It would be a significant advance in the art of medicinal biology to identify this enzyme or combination of enzymes, for the purpose of further elucidating the retroviral lifecycle and thereby providing new information for developing antiviral drugs. In addition, such enzymes themselves would be of particular utility as targets for drug development, inasmuch as the propensity of cellular enzymes to mutate in response to an external stimulus is many-fold reduced as compared with the propensity of the virus to mutate.

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SUMMARY OF THE INVENTION

According to one aspect of the present invention, a method of identifying cellular enzymes needed to complete integration of retroviral DNA into a genome is provided. The method comprises: (a) providing a pair of retroviral vectors, one of the pair encoding a functional integrase and the other of the pair encoding a non-functional integrase; (b) providing samples of a cell line suspected of lacking at least one enzyme needed to complete integration of the retroviral vector into the genome of the cell line; (c) infecting one of the samples with the retroviral vector encoding the functional integrase and another of the samples with the retroviral vector encoding the non-functional integrase; and (d) measuring cell death in each of the samples, a greater amount of cell death in the sample infected with the retroviral vector encoding the functional integrase, as compared with the sample infected with the retroviral vector encoding the non-functional integrase, being indicative that the enzyme lacking in the cell line is needed to complete integration of the retroviral DNA into the cell's genome. Test kits for carrying out the method are also provided.

According to another aspect of the invention, a method is provided for identifying an agent that interferes with a retroviral life cycle at a step preceding completion of integration of retroviral DNA into a host cell genome. The method comprises: (a) providing a cell line that is unable to perform a DNA repair step in integration of retroviral DNA into the cell's genome; (b) infecting with a retrovirus one sample of the cell line in the presence of a test compound suspected of interfering with the retroviral life cycle; (c) infecting with a retrovirus an equivalent sample of the cell line in the absence of the test compound; and

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(d) measuring cell death of the infected samples of cell lines, a decrease in amount of cell death in the presence of the test compound as compared with in the absence of the test compound being indicative that the test compound is an agent that interferes with the retroviral life cycle at a step preceding completion of retroviral DNA integration into the cell's genome. In a preferred embodiment, the cell line comprises a non-functional DNA-dependent protein kinase. Most preferably, the cell line is derived from a *scid* mouse.

A test kit for carrying out this method is also provided.

According to another aspect of the present invention, a method is provided for interfering with retroviral replication in a host cell infected with the virus. The method comprises inhibiting an enzyme of the host cell needed to perform the repair step of retroviral DNA integration into the host cell genome. Preferably, the enzyme is a DNA-dependent protein kinase. In a preferred embodiment, the inhibiting is accomplished by exposing the cell to an exogenous agent that inhibits the enzyme.

Other features and advantages of the present invention will be understood by reference to the drawings and descriptions thereof, detailed description and examples which follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Set of histograms showing that retroviral infection is lethal to DNA-PK_{cs} deficient, *scid* pre-B cells.

Figure 2. Schematic diagram of a retroviral DNA construct containing an expressible neo reporter gene, RCASPB-M(neo).

Figure 3. Set of histograms showing that killing of *scid* cells by a retroviral vector requires an

active integrase. Below the histograms are shown two retroviral constructs. The upper construct encodes an active integrase, whereas the lower construct contains a mutation that produces a single amino acid substitution (D64E) in the active site that renders the integrase inactive.

Figure 4. Histogram showing that retroviral infection induces the apoptotic pathway in *scid* cells, and flow chart showing the basic steps in the apoptosis pathway.

Figure 5. Histograms showing activation of DNA-PK_{CS} in human (HeLa) cell nuclear extracts. Cells were exposed to different treatments, then lysed. The p53 peptide serves as a substrate for phosphorylation by DNA-PK. The histogram on the left shows the result of treating cells with sheared DNA to activate the lysate. The histogram on the right shows the results of infecting or mock-infecting cells with a retroviral vector. No additional exogenous DNA was added.

Figure 6. Graphs and histogram showing that DNA-PK_{CS} deficient *scid* cells are killed by HIV-based vector infection. The graphs on the left show percent viability over time. The histogram on the right shows β -galactosidase activity in vector-infected cultures.

Figure 7. Histogram showing that AZT protects *scid* cells from virus-mediated cell killing.

Figure 8. Histogram showing the effect of Wortmannin on retroviral integration of HeLa cells (G418 resistant colonies).

Figure 9. Graph showing the effect of Wortmannin on growth of HeLa cells.

Figure 10. Graph showing the effect of Wortmannin on DNA-PK-related activity.

Figure 11. Graph showing the effect of Wortmannin on viability of S33 cells. *Scid* cells (S33

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line) were treated with 0-2 μ M Wortmannin and simultaneously infected with the RCASPB-M virus as described in Example 1.

5 DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has now been discovered that the final step in retroviral DNA integration (the "repair" step) requires components of the cellular DNA double strand break (DSB) repair
10 pathway. Specifically, DNA-dependent protein kinase (DNA-PK) activity is necessary to complete the integration of viral DNA into the host genome.

DNA-PK is composed of a DNA binding component (Ku) and a large catalytic subunit (DNA-PK_{cs}). DNA-PK
15 functions in DNA double strand break (DSB) repair and V(D)J recombination involved in generation of antibody diversity (see Lees-Miller, Biochem. Cell Biol. 74: 503-512, 1996). It is now known that the severe combined immunodeficient (*scid*) mouse, and cell lines derived
20 therefrom, produce a defective DNA-PK comprising a nonsense mutation in the carboxyl terminal region of the catalytic subunit (Blunt et al., Proc. Natl. Acad. Sci. USA 93: 10285-10290, 1996). *Scid* mice fail to develop mature T and B lymphocytes because their defective DNA-PK
25 is unable to carry out the functional rearrangement of the elements encoding the immunoglobulin and T-cell receptor genes (i.e., V(D)J recombination). *Scid* mice also are exquisitely sensitive to ionizing radiation, which is known to cause double strand DNA breaks. This
30 sensitivity also is believed to arise from their lack of a functional DNA-PK to perform the DSB repair.

The present inventors infected mouse cell lines derived from *scid* or normal mice with one of three different retroviral vectors, derived from ASLV, MoMLV
35 and HIV, respectively. Infection with these viruses

induced cell death in *scid*, but not normal cells. Cell death was not inducible by heat-inactivated virus. To determine whether the observed cell death was due to retroviral integration, cells were infected with one of a pair of avian retroviral vectors, RCA8KSneo and RCAD64Eneo. Sequences of these viral DNAs are identical, except RCAD64Eneo carries a mutation that encodes an amino acid substitution that inactivates only the integrase protein. RCA8KSneo was found to induce cell death in *scid* cells, whereas the integrase-defective RCAD64Eneo did not. These results, along with additional experimental results set forth in the examples, demonstrate that (1) cell death in *scid* lines is due to retroviral integration; (2) cell death occurs via an apoptosis pathway; and (3) DNA-PK_{cs} is needed for retroviral integration, at the "repair" step, which IN fails to catalyze *in vitro*. It is possible that other cellular enzymes are involved in the repair step of viral integration, and that IN participates in this process; however, the foregoing results demonstrate the necessity of a functional DNA-PK to complete the integration.

In addition to the inventors' discovery of the requirement for DNA-PK to complete retroviral integration, the inventors have further discovered that a certain percentage of a cell population may catalyze the repair step of viral integration by an alternative mechanism, which involves the ATM (Ataxia-telangiectasia mutated) kinase. This discovery is described in greater detail in Example 7. Accordingly, the ATM kinase may serve as a secondary target for antiviral drug development, as discussed in greater detail below.

The above-summarized discoveries made in accordance with the present invention enable advances in anti-retroviral drug discovery in two significant areas: (1) rapid and convenient assays are now possible to

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screen for drugs that inhibit early steps in the retrovirus lifecycle, i.e., steps up to and including the processing and joining steps catalyzed by IN; and (2) a cellular enzyme (DNA-PK) involved in the retroviral life
5 cycle is now available as a target for drug development.

The present invention includes several types of assays. For instance, as mentioned above, other cellular enzymes besides DNA-PK may be needed or involved in the repair step of retroviral integration, but regardless of
10 the number of enzymes involved in the repair step, failure of a cell to carry out this step results in cell death. Accordingly, in one type of assay of the present invention, paired retroviral vectors, such as RCA8KSneo and RCAD64Eneo, in which one member of the pair is
15 integrase-defective, can be used in combination with knock-out or mutant cell lines for the rapid identification of such cellular genes involved in retroviral integration. The integrase-functional retroviral vector will induce cell death in cells
20 deficient for a gene needed for retroviral integration. If the gene is truly required for integration, infection with the integrase-defective retroviral vector will have no effect on the test cell line.

Due to the genetic similarity among retroviral
25 genomes, paired vectors such as RCA8KSneo and RCAD64Eneo (which are ASLV derivatives) can be constructed for any retrovirus. Retroviruses suitable for such modification include, but are not limited to, HIV, SIV, MoMLV, HTLV, FeLV, BIV and EIAV, among others.

30 In another type of assay, a *scid* cell line, or any other cell line carrying a defect that results in failure to carry out the repair step of retroviral integration, is used in a rapid screen for agents capable of impeding or inhibiting steps in the retroviral life
35 cycle, up to and including the processing and joining

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steps of integration catalyzed by IN. This includes blocking the function of either viral or cellular genes required during the early stages of viral propagation.

5 In a preferred embodiment, *scid* cells are used in assays of this type. However, other DNA-PK defective cell lines also may be used (such as the rodent mutant cell line, *xrs-5*, described by Lees-Miller et al., Science 267: 1183-1185, 1995), as may be any cell line carrying a defect that causes failure to perform the
10 repair step of retroviral integration and thus trigger apoptosis. Indeed, useful cell lines of this type can be identified using the paired retrovirus assay described in the preceding paragraphs.

In the simplest form of this screening assay,
15 an integration repair-defective cell line, such as a *scid* line, is infected with a replication-competent retroviral vector in the presence or absence of a test compound suspected of blocking early steps in the retrovirus lifecycle. If the test compound indeed does impede or
20 block one or more of these early events, the cells will continue to live. If the compound is ineffective, the cells will die. This assay offers the distinct advantage of a positive selection - the cells live if the candidate drug is effective, but die if it is not.

25 To demonstrate the efficacy of this assay, *scid* cells and normal cells were infected with virus in the presence or absence of AZT, a known inhibitor of reverse transcription. Inhibition of any step prior to integration is predicted to reverse killing of *scid* cells
30 by the retrovirus. The results shown in Figure 7 demonstrate that AZT abrogates the cell-killing effect of retrovirus infection, as expected.

The screening assay can be refined through the use of appropriate controls. For instance, assays
35 preferably are constructed so that the integration

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repair-defective cell lines are compared with equivalent control cell lines that are competent in integration repair. In a preferred embodiment, *scid* cells and a near-isogeneic normal cell line are used. Another useful
5 control is side-by-side infection with a paired set of retroviral vectors as described above.

In any of the foregoing assays, cell death can be measured in a variety of ways. One way is simply to measure cell death, e.g., by trypan blue staining.
10 Another way is to measure expression of a selectable marker gene or a reporter gene, the expression of which is dependent on stable integration of the viral vector. As defects in the integration repair step appear to cause cell death via the apoptosis pathway, a preferred method
15 comprises measuring an indicator of apoptosis. A particularly preferred embodiment utilizes measurement of caspase activation, which is an early event in apoptosis.

The present invention also identifies DNA-PK as a novel gene target for inhibition of retroviral
20 replication. Of particular interest in this regard is the inhibition of HIV replication, as a new therapy for AIDS. Current anti-HIV therapies target HIV proteins, predominantly HIV reverse transcriptase and protease. However, rapid emergence of resistance to these drugs is
25 well documented, due to the ability of the virus to rapidly mutate.

DNA-PK is a cellular gene and does not mutate with the frequency of HIV. Accordingly, inhibitors of DNA-PK could impede or interrupt HIV replication, and the
30 probability of resistance developing to such inhibitors would be comparatively low as compared to inhibitors of viral genes. DNA-PK inhibition is targeted specifically to the cells desired to be eliminated - newly infected cells. If DNA-PK is inhibited infected cells will die.
35 However, virus spread will be blocked since the cells

will die prior to virus production.

Thus, inhibition of DNA-PK is expected to be an extremely useful AIDS therapy. DNA PK_{cs} can be inhibited directly by DNA-PK inhibitors, several of which are
5 currently available (e.g., single stranded DNA, pyrophosphate, 6-dimethyl-aminopurine and the pyridone derivative, OK-1035 (Take et al., Biochem. Biophys. Res. Comm. 215: 41-47, 1995). In addition, as described in the examples, the inventors have shown that Wortmannin
10 inhibits retroviral integration in HeLa cells by inhibiting DNA-PK activity in those cells.

Alternatively, new inhibitors can be developed. DNA-PK_{cs} also can be inhibited indirectly. A recent report indicates that some growth regulators can down-
15 regulate DNA-PK activity (Bandyopadhyay et al., J. Biol. Chem. 273: 1568-1573, 1998). Inhibition of DNA-PK by growth factors may be especially advantageous for anti-HIV therapy due to the low toxicity of growth factors.

20 The following examples are provided to describe the invention in greater detail. These examples are intended to illustrate and not to limit the invention.

25

EXAMPLE 1
Lethality of Retroviral Infection to
DNA-PK_{cs} Deficient Scid Pre-B Cells

The virus used for the experiments described
30 below is an avian retroviral vector that is replication-competent in avian cells. A schematic diagram of the vector is shown in Figure 1. As indicated in the diagram, the vector includes a murine amphotrophic envelope gene that allows the virus to infect and
35 integrate its DNA in a wide variety of mammalian cells, including mouse and human cells.

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Three independently-derived mouse *scid* pre-B cell lines (S7, S29 and S33) were used, as well as an isogeneic normal cell line. The cell lines were infected with the retroviral vector RCASBP-M (moi approx. 2) and
5 1:2 and 1:10 dilutions thereof. Controls were (1) mock infection and (2) infection with heat-inactivated virus). Cell death was measured at various time points following infection, using trypan blue staining.

As can be seen from the results shown in Figure
10 1, cell viability dropped dramatically after infection at a multiplicity of approximately two infectious units per cell. No loss of viability was seen upon mock infection or infection with heat-inactivated virus. The loss of
15 viability was dependent upon virus concentration. A reduced effect was observed in cells infected with a 1:2 dilution of the virus, and almost no killing was observed in cells infected with a 1:10 diluted virus. Viability of the normal cells was unaffected by infection with the virus.

20 These results indicate that infection by a retrovirus is lethal to *scid* cells.

EXAMPLE 2

25 Expression of a neo Reporter Gene in Vector-Infected *Scid* Fibroblasts

To verify that the cell killing described in Example 1 was due to the defect in DNA-PK and not some other anomaly of transformed *scid* lymphocyte cell lines,
30 an experiment was performed to determine if the retroviral vector can become stably integrated in primary *scid* fibroblasts.

For these experiments, the avian retroviral vector described in Example 1 was modified to contain a
35 neo selectable marker gene, as shown in Figure 2 (RCASBP-M(neo)). Fibroblasts having a stably integrated

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retroviral vector will be resistant to the antibiotic, G418. Cells (sixth passage) were plated at a density of 1×10^5 per 60 mm dish. The following day, cells were infected with virus. Neo+ colonies were selected by
5 addition of 500 $\mu\text{g/ml}$ G418, 24 hours later. Results are shown in Table 1 below.

TABLE 1

10	<u>Virus Dilution</u>	<u>Normal Fibroblasts (Number of Colonies)</u>	<u>Scid Fibroblasts (Number of Colonies)</u>
15	10^{-3}	125, 82	9, 7
	10^{-4}	26, 34	2, 0
20	Mock	0, 0	0, 0

As can be seen in Table 1, no G418-resistant colonies grew on mock-infected plates. Moreover, the number of resistant colonies was dependent on virus concentration. In addition, the number of resistant
25 colonies on plates containing normal fibroblasts was ten times higher than the number of resistant colonies on *scid* fibroblasts.

The simplest interpretation of these data is that there were fewer G418 resistant colonies on the *scid*
30 fibroblast plates because, as with the lymphocytes described in Example 1, most of the infected cells were killed. Such killing is expected if the partially integrated viral DNA is recognized as DNA damage by the cell, but cannot be repaired by the cellular repair
35 mechanism due to the fact that DNA-PK is inactive in these cells.

EXAMPLE 3

5 **Demonstration that Killing of *Scid* Cells by the
 Retroviral Vector Requires an Active Integrase**

Two additional derivatives of the avian
retroviral vector described in Example 1 were constructed
10 for the purpose of determining if integration, *per se*, is
the critical factor for the lethality of retroviral
infection observed in *scid* cell lines. These vectors are
shown schematically in Figure 3. In one vector, a DNA
segment was substituted into the integrase gene that
15 included a mutation in the active site of the enzyme.
The resulting conservative substitution in the D, D(35)E
motif inactivates the integrase, but does not affect any
other early events in the virus lifecycle. In a control
vector, the same DNA segment was substituted with a wild-
20 type sequence.

These retroviral vectors were used to infect
normal and *scid* pre-B cells, as described in Example 1.
Cell viability was measured as described in Example 1.

Results are shown in Figure 3. The control
25 virus was found to be just as lethal to *scid* cells, but
not control cells, as the parental vector. By contrast,
the integrase-defective virus had no effect on *scid* cell
viability. These results indicate that the lethal event
in retroviral infection of *scid* cells is integration.
30 Normal cells survive because integration can be completed
with the help of the same repair functions that are
critical for DNA strand break repair and V(D)J
recombination, i.e., DNA-PK.

35

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EXAMPLE 4
Demonstration that Retroviral Infection
Induces the Apoptotic Pathway in *Scid* Cells

5 In *scid* cells, DNA damage is known to induce apoptosis. This pathway involves a cascade of events (summarized in Figure 4) that result in visible changes in the cell. Visual inspection of retrovirus-infected *scid* cells suggested that they were dying by apoptosis.

10 To quantify the effect, induction of a characteristic ICE family protease, caspase 3, was measured in cell lysates.

 Control cells and *scid* cells were infected with retrovirus in the presence or absence of DEVD-fmk, a synthetic tetrapeptide that inhibits caspase activity

15 specifically and irreversibly. Normal or *scid* cells were infected with the virus, lysed 16 hours later, and caspase activity measured fluorimetrically by formation of a reaction product of the caspase substrate, DEVD-pNA (p-nitroanilide). Lysates were normalized by initial

20 cell concentration and protein content.

 Results are shown in Figure 4. A significant increase in caspase activity (approximately 4X) was observed in the infected as compared to uninfected *scid* cells. The specificity of the reaction was verified by

25 addition of the specific inhibitor, which brought the value down to the level seen in the uninfected cells. There appeared to be a slight increase in activity in infected normal cells as well, but this activity was no greater than the background level in the *scid* cells.

30 These results indicate that retrovirus-infected *scid* cells die by apoptosis. Other experimental results that demonstrate that retrovirus-infected *scid* cells die by apoptosis are set forth by Daniel et al., Science 284: 644-647, 1999 (incorporated by reference herein).

35

EXAMPLE 5

Demonstration that DNA-PK Kinase Activity is Induced by Retroviral Infection of HeLa Cells

5 The ability of retroviral infection to actually induce DNA-PK kinase activity was determined. Standard DNA-PK assays cannot detect DNA-PK activity in rodent cells. However, the activity can be detected in human (e.g., HeLa) cells because they contain about 50-fold
10 greater amounts of the enzyme than do rodent cells. Accordingly, human HeLa cells were used for the DNA PK kinase assay described below.

A p53 peptide serves as a specific substrate for phosphorylation by DNA-PK (Bandyopadhyay et al., J. Biol. Chem. 273: 1568-1573, 1998). Using this substrate,
15 HeLa cells were either treated with exogenous sheared DNA (known to activate DNA-PK, or they were infected with the retroviral vector. Controls were (1) cells not exposed to exogenous sheared DNA and (2) mock-infected cells.

20 Results are shown in Figure 5. The panel on the left side shows results of the assay for DNA-PK activity in the presence or absence of sheared salmon sperm DNA. As can be seen, the kinase was activated in these control experiments by addition of the exogenous
25 DNA. It is clear that the activity in this assay depends on all three components of the assay system - p53 peptide substrate, exogenous sheared DNA, and HeLa cells.

In the experiments shown in the panel on the right, nuclear extracts were prepared at various times
30 after infection of HeLa cells with the avian retroviral vector. In these assays, no exogenous DNA was added to the assay mixture. As can be seen, DNA-PK activity increased about 2.5-fold by eight hours after infection. This corresponds to the time that integration events
35 typically are observed in infected cells. Thus, these results indicate that DNA-PK is activated in response to infection with retrovirus.

EXAMPLE 6**Demonstration that *Scid* Cells are Killed by Infection with an HIV-Based Retroviral Vector**

5 The experiments described in the foregoing examples were performed using an avian retroviral vector. To determine if the observed responses can be generalized to other retroviruses, *scid* cells were infected with a
10 murine leukemia virus and with an HIV-based vector that was pseudotyped with the VSV G protein to allow it to enter rodent cells.

 Figure 6 shows the results of the experiments with the HIV-based vector, which were similar to the
15 results observed with the murine virus. As can be seen, cells infected with the HIV vector, at a multiplicity of about one infectious unit per cell, showed a loss of viability over time, while mock-infected cells or cells infected with heat-inactivated vector did not. Moreover,
20 normal cells were not affected by any treatment.

 The HIV vector used included a β -galactosidase reporter gene that must be integrated into the chromosome to be expressed. As shown in Figure 6, measurement of β -galactosidase activity in lysates of infected cells shows
25 that the *scid* lines had only 30-50% of the activity of the normal cell line. Taken together, these results indicate that lethality to DNA-PK deficient *scid* cells after retroviral infection (at the integration step) is a generalized effect of retroviruses.

EXAMPLE 7**Effects of Wortmannin**

30 Retroviral integration in HeLa cells. The
35 effect of Wortmannin on retroviral integration in HeLa cells (G418 resistant colonies) was investigated. HeLa cells were plated at 10^5 cells per 60 mm dish and infected

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with the IN+ virus that carries a neo marker, which was described in Example 2. Neo+ colonies were selected by addition of 1 mg/ml G418, 24 hours later.

Results are shown in Figure 8. As can be seen, Wortmannin inhibits retroviral integration in HeLa cells in a concentration-dependent manner. Integration is essentially abrogated with 20 μ M Wortmannin.

Growth of HeLa cells. HeLa cells were plated at a concentration of 10^5 cells per 60 mm dish. Cells were exposed to various concentrations of Wortmannin and counted three days after exposure. Results are shown in Figure 9. As can be seen, Wortmannin had no effect on the growth of HeLa cells in the 1-20 μ M range, even though Wortmannin inhibits retroviral integration in the cells at that concentration range (Figure 8).

DNA-PK-related activity. HeLa cells were treated overnight with Wortmannin. After that, cells were lysed and a DNA-PK activity assay was performed as described in earlier examples (see also Daniel et al., 1999, *supra*). Results are shown in Figure 10. As can be seen, Wortmannin inhibits DNA-PK activity in HeLa cells. Inhibition of the DNA-PK-related kinase activity correlates with inhibition of retroviral integration.

Viability of S33 cells. DNA-PK kinase activity is the primary target of the DNA-PK inhibitor Wortmannin *in vivo*. However, Wortmannin also inhibits other DNA-PK related kinases, such as ATM, though at high concentrations.

A percentage of *scid* cells survive infection (Example 1 and Daniel et al., 1999, *supra*). To determine if this "leakiness" is due to a DNA-PK-related kinase, *scid* cells were treated with Wortmannin and infected with virus (RCASB-M; see Figure 2). Results are shown in Figure 11. As can be seen, at 2 μ M, Wortmannin enhances the lethal-effect of the virus. This finding suggests

that a DNA-PK-like kinase is responsible for the leakiness of the virus-mediated *scid* cell killing.

Inhibition of G418 colony formation in ATM cell lines. About 10% of the DNA-PK-deficient *scid* cells are susceptible to retroviral infection and integration. Results shown in Figure 11 suggest that this leakiness is due to an alternative pathway that is mediated by a DNA-PK-like kinase. The ATM kinase is closely related to DNA-PK and also plays a role in DNA repair. It was therefore investigated whether ATM could be involved in an alternative pathway of retroviral integration.

Retrovirus (IN+ - see Figure 3) can still integrate into MO59J cells, which have no DNA-PK (see Table 2 below). These data confirm that the above mentioned "leakiness" is not due to a residual DNA-PK activity. Treatment of MO59J cells (DNA-PK null) with Wortmannin confirms the alternative pathway is Wortmannin-sensitive and thus involved DNA-PK-related kinase, but not DNA-PK. This finding is consistent with the results presented in Figure 11.

The primary target of Wortmannin is DNA-PK kinase. The ATM kinase is inhibited at higher Wortmannin concentrations than those required to inhibit the DNA-PK kinase. Cells deficient in the ATM kinase were infected with the IN+ virus carrying a *neo* marker, then treated with Wortmannin. Results are shown in Table 2.

TABLE 2

<u>cells</u>	<u>HeLa</u>	<u>AT2SF</u>	<u>MO59J</u>	<u>HeLa</u>	<u>AT5B1</u>	<u>CHO-K1</u>	<u>XR-1</u>
DNA-PK	+	+	-	+	+	+	-(XRCC4-)
ATM	+	-	+	+	-	+	+
<u>Wort. (μM)</u>	<u>percentage of G418^r colonies</u>						
0	100	100	100	100	100	100	100
1	56	11	43	65	4	77	66
5	45	5	35	24	1	35	44

As shown, treatment with Wortmannin led to a dramatic reduction in retroviral integration in ATM cells, much faster than in HeLa cells (Table 2 shows two different experiments with HeLa cells and two ATM-deficient lines, 5 AT2SF and AT5B1). It is concluded that ATM is involved in the alternative pathway of retroviral integration. Moreover, as can be seen from the Table, G418 colony formation in XRCC4- cells was still sensitive to Wortmannin. Thus, the XRCC4 gene, which is a part of the 10 DNA-PK pathway, appears not to be involved in the alternative pathway.

The present invention is not limited to the embodiments described and exemplified above, but is 15 capable of variation and modification without departure from the scope of the appended claims.

What is claimed:

1. A method of identifying cellular enzymes
needed to complete integration of retroviral DNA into a
5 genome, which comprises:
 - a) providing a pair of retroviral vectors,
one of the pair encoding a functional integrase and the
other of the pair encoding a non-functional integrase;
 - b) providing samples of a cell line
10 suspected of lacking at least one enzyme needed to
complete integration of the retroviral vector into the
genome of the cell line;
 - c) infecting one of the samples with the
retroviral vector encoding the functional integrase and
15 another of the samples with the retroviral vector
encoding the non-functional integrase; and
 - d) measuring cell death in each of the
samples, a greater amount of cell death in the sample
infected with the retroviral vector encoding the
20 functional integrase, as compared with the sample
infected with the retroviral vector encoding the non-
functional integrase, being indicative that the enzyme
lacking in the cell line is needed to complete
integration of the retroviral DNA into the cell's genome.
25
2. The method of claim 1, wherein the cell
line is a mouse cell line.
3. The method of claim 1, wherein the pair of
30 retroviral vectors are derived from a retrovirus selected
from the group consisting of ASLV, MoMLV and HIV.
4. The method of claim 3, wherein the pair of
retroviral vectors are RCA8KSneo and RCAD64Eneo.
35

5. The method of claim 1, which further comprises providing samples of a control cell line capable of completing the integration of the retroviral DNA.

5

6. The method of claim 1, wherein the cell death is measured by determining the percentage of viable cells remaining in the samples after the infecting.

10

7. The method of claim 1, wherein the cell death is measured by measuring a cellular component of the apoptosis pathway.

15

8. The method of claim 7, wherein the cellular component comprises a caspase.

20

9. The method of claim 1, wherein the pair of retroviral vectors further comprises a selectable marker gene that confers resistance only if the retroviral vector is stably integrated into the cell's genome, and the cell death is measured by plating the infected cells on selection medium and thereafter counting colonies on the medium.

25

10. The method of claim 1, wherein the pair of retroviral vectors further comprises a reporter gene that is expressed only if the retroviral vector is stably integrated into the cell's genome, and the cell death is measured by detecting the product of the reporter gene.

30

11. A test kit for identifying cellular enzymes needed to complete integration of retroviral DNA into a genome, which comprises a container containing:

35

a) a pair of retroviral vectors, one of the pair encoding a functional integrase and the other of

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the pair encoding a non-functional integrase; and

b) instructions for using the pair of retroviral vectors to identify the cellular enzymes.

5 12. The test kit of claim 11, which further comprises a control cell line capable of completing the integration of the retroviral DNA.

10 13. The test kit of claim 11, which further comprises reagents for measuring the cell death.

15 14. A method of identifying an agent that interferes with a retroviral life cycle at a step preceding completion of integration of retroviral DNA into a host cell genome, which comprises:

a) providing a cell line that is unable to perform a DNA repair step in integration of retroviral DNA into the cell's genome;

20 b) infecting with a retrovirus one sample of the cell line in the presence of a test compound suspected of interfering with the retroviral life cycle;

c) infecting with a retrovirus an equivalent sample of the cell line in the absence of the test compound; and

25 d) measuring cell death of the infected samples of cell lines, a decrease in amount of cell death in the presence of the test compound as compared with in the absence of the test compound being indicative that the test compound is an agent that interferes with the retroviral life cycle at a step preceding completion of
30 retroviral DNA integration into the cell's genome.

35 15. The method of claim 14, wherein the cell line comprises a non-functional DNA-dependent protein kinase.

- 25 -

16. The method of claim 15, wherein the cell line is derived from a *scid* mouse.

17. The method of claim 14, wherein the
5 retrovirus is selected from the group consisting of ASLV, MoMLV and HIV.

18. The method of claim 14, which further
comprises infecting in the presence and absence of the
10 compound a control cell line capable of completing the integration of the retroviral DNA.

19. The method of claim 14, which further
comprises infecting additional samples of the cell line
15 with a retrovirus which produces a non-functional integrase.

20. The method of claim 14, wherein the cell
death is measured by determining the percentage of viable
20 cells remaining in the samples after the infecting.

21. The method of claim 14, wherein the cell
death is measured by measuring a cellular component of
the apoptosis pathway.

25

22. The method of claim 21, wherein the
cellular component comprises a caspase.

23. The method of claim 14, wherein the genome
30 of the retrovirus further comprises a selectable marker gene that confers resistance only if the retroviral DNA is stably integrated into the cell's genome, and the cell death is measured by plating the infected cells on selection medium and thereafter counting colonies on the
35 medium.

- 26 -

24. The method of claim 14, wherein the genome of the retrovirus further comprises a reporter gene that is expressed only if the retroviral DNA is stably integrated into the cell's genome, and the cell death is measured by detecting the product of the reporter gene.

25. A test kit for identifying an agent that interferes with a retroviral life cycle at a step preceding completion of integration of retroviral DNA into a host cell genome, which comprises:

- a) a cell line that is unable to perform a DNA repair step in integration of retroviral DNA into the cell's genome;
- b) a retroviral vector; and
- c) instructions for using the cell line and the vector to identify agents that interfere with the retroviral life cycle.

26. The test kit of claim 25, wherein the cell line is derived from a *scid* mouse.

27. The test kit of claim 25, which further comprises a control cell line capable of completing the integration of the retroviral DNA.

28. The test kit of claim 25, which further comprises a control retroviral vector that encodes a non-functional integrase.

29. The test kit of claim 25, which further comprises reagents for measuring the cell death.

30. A method of interfering with retroviral replication in a host cell infected with the virus, which comprises inhibiting an enzyme of the host cell needed to

- 27 -

perform the repair step of retroviral DNA integration into the host cell genome.

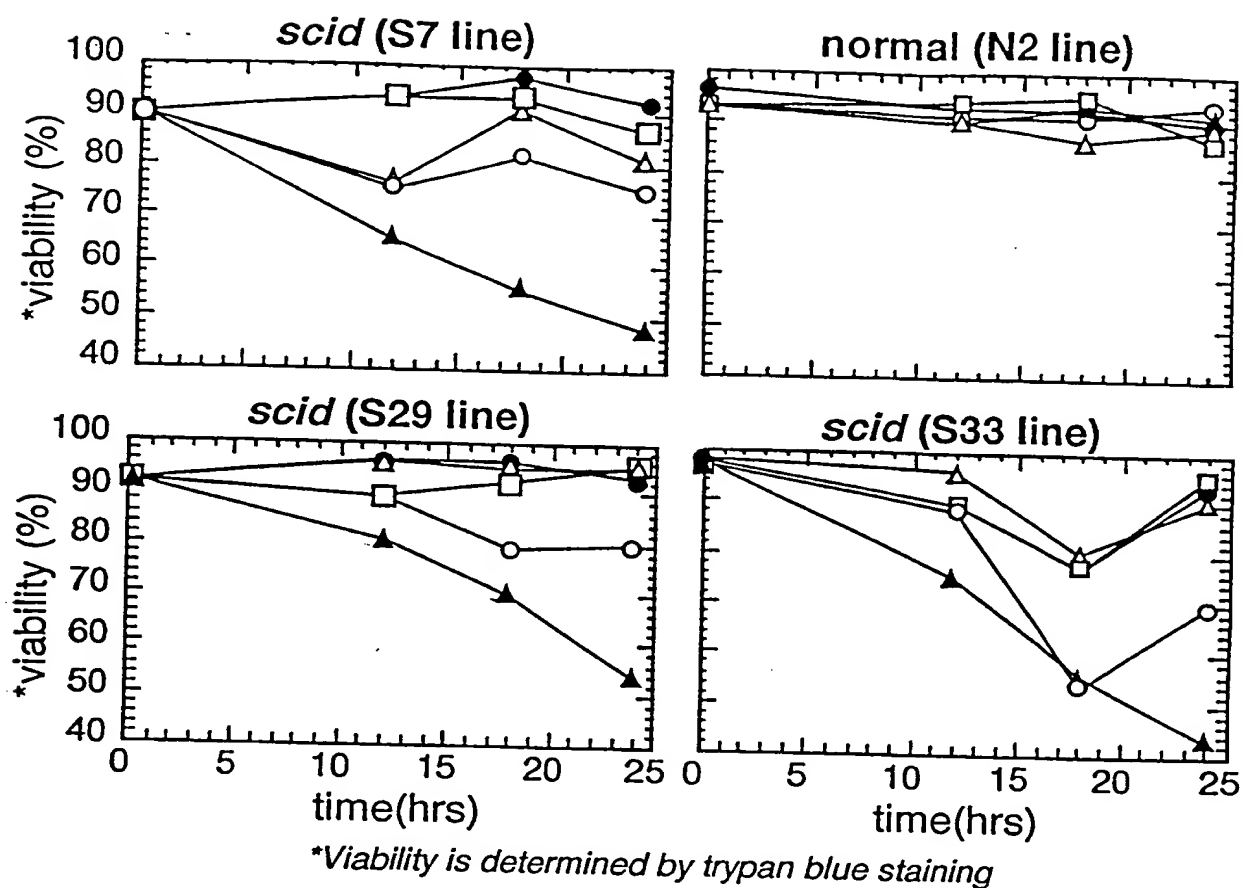
31. The method of claim 30, wherein the enzyme
5 is a DNA-dependent protein kinase.

32. The method of claim 30, wherein the enzyme is an Ataxia telangiectasia mutated kinase.

10 33. The method of claim 30, wherein the host cell is a human cell.

34. The method of claim 30, wherein the inhibiting is accomplished by exposing the cell to an
15 exogenous agent that inhibits the enzyme.

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- ▲ RCASBP-M (moi≈2)
- RCASBP-M (1:2 dilution)
- △ RCASBP-M (1:10 dilution)
- mock infection
- heat-inactivated RCASBP-M

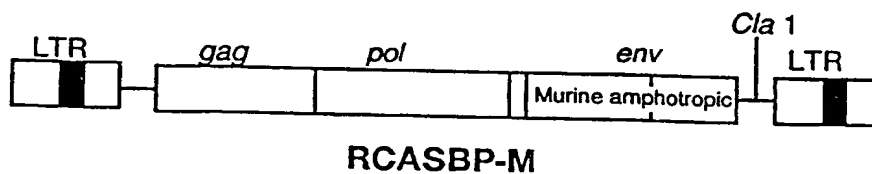


Figure 1

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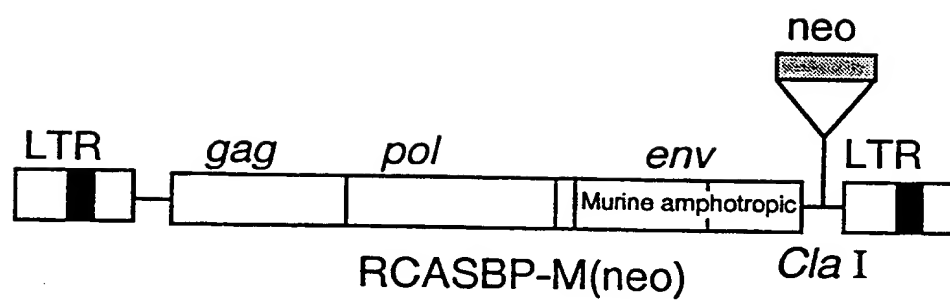


Figure 2

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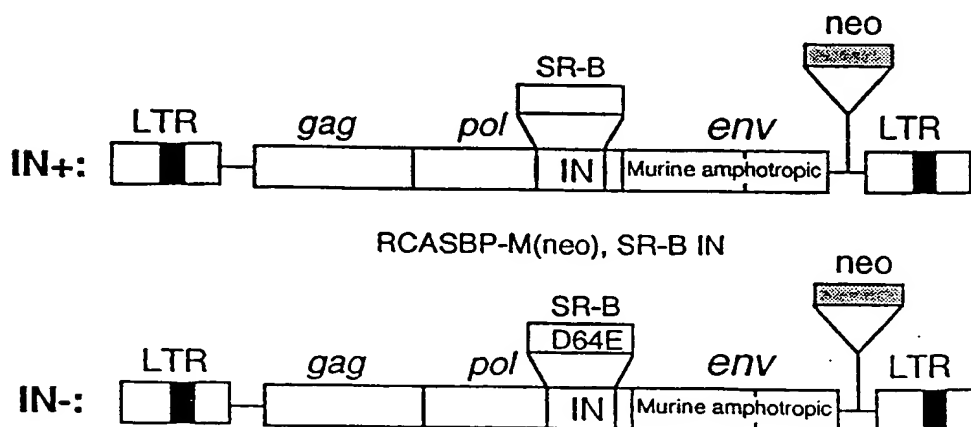
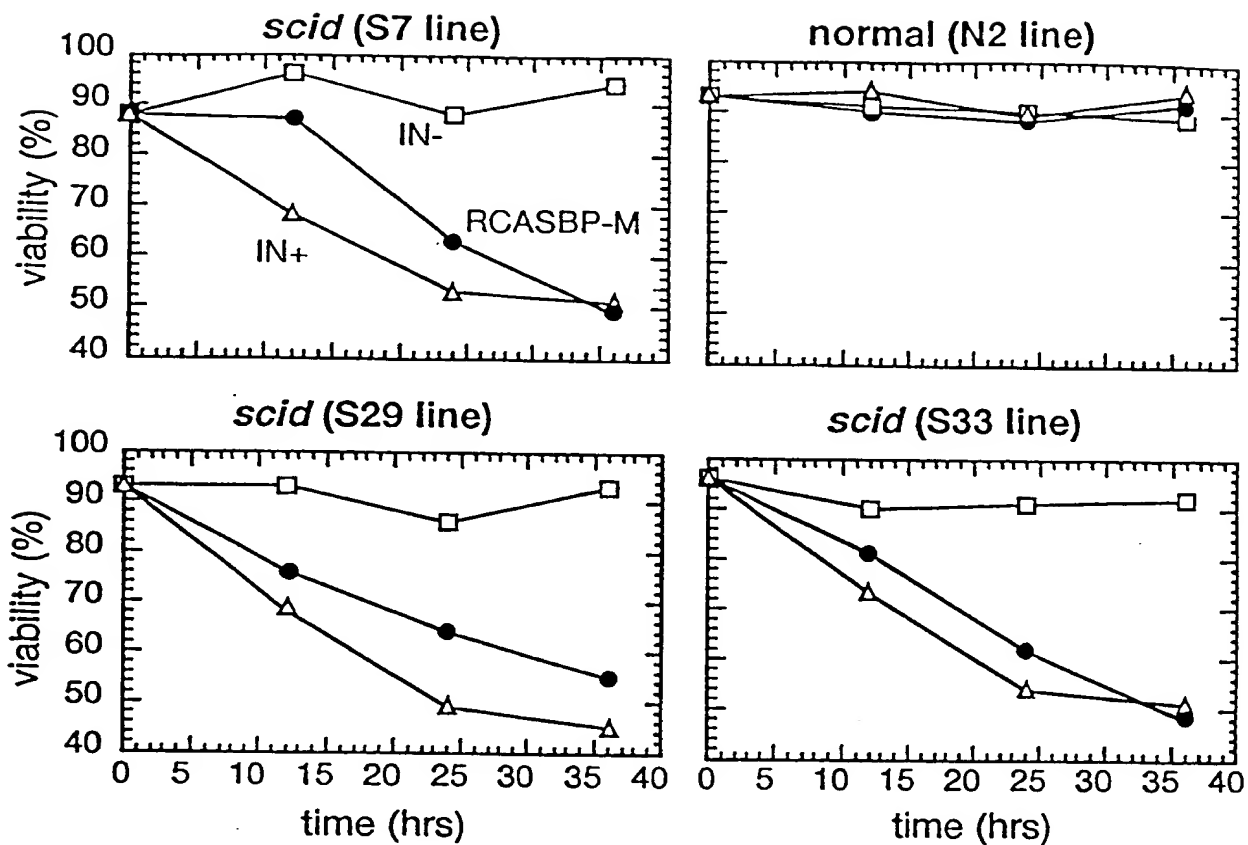


Figure 3

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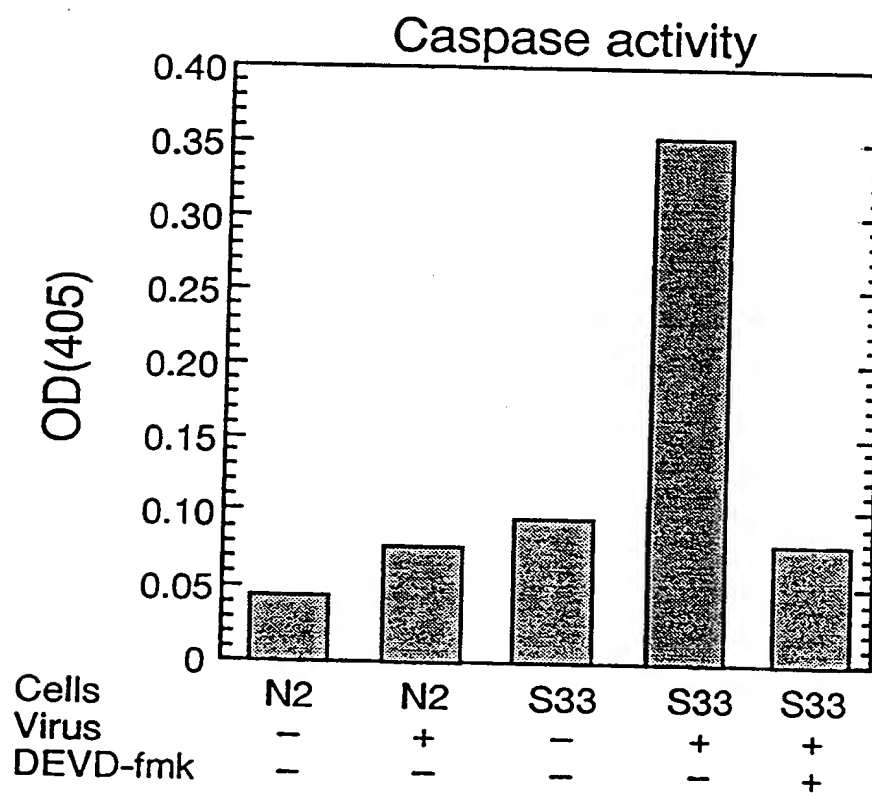
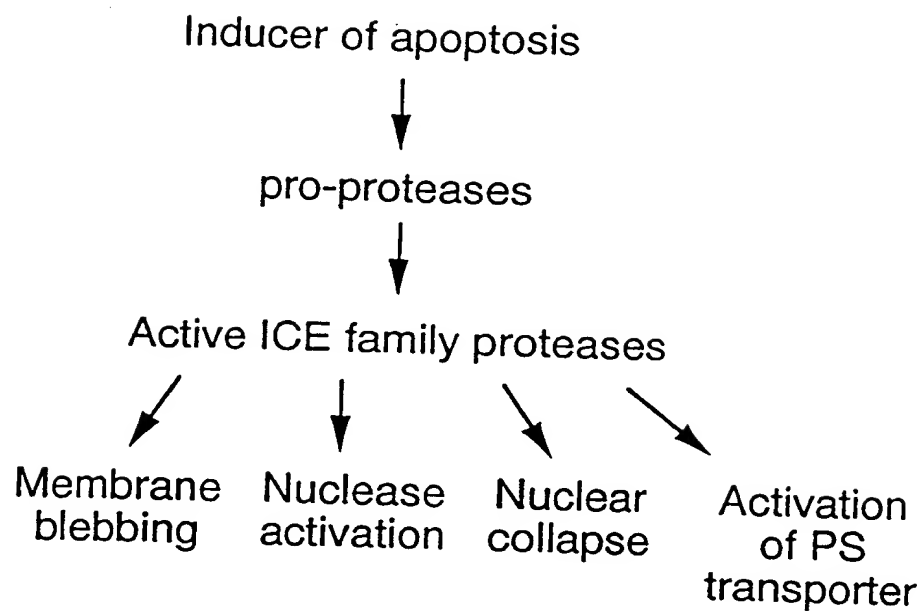
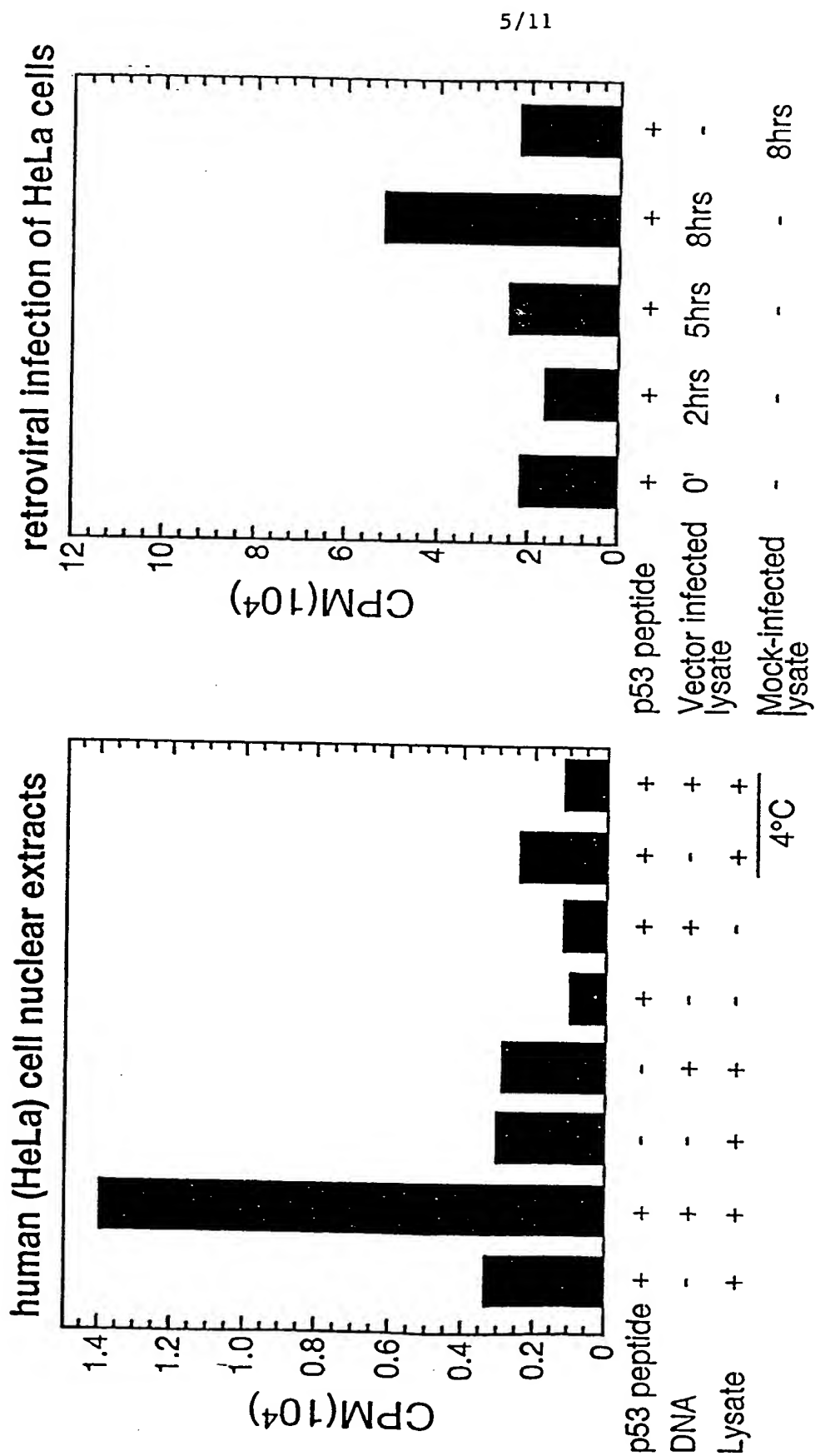


Figure 4



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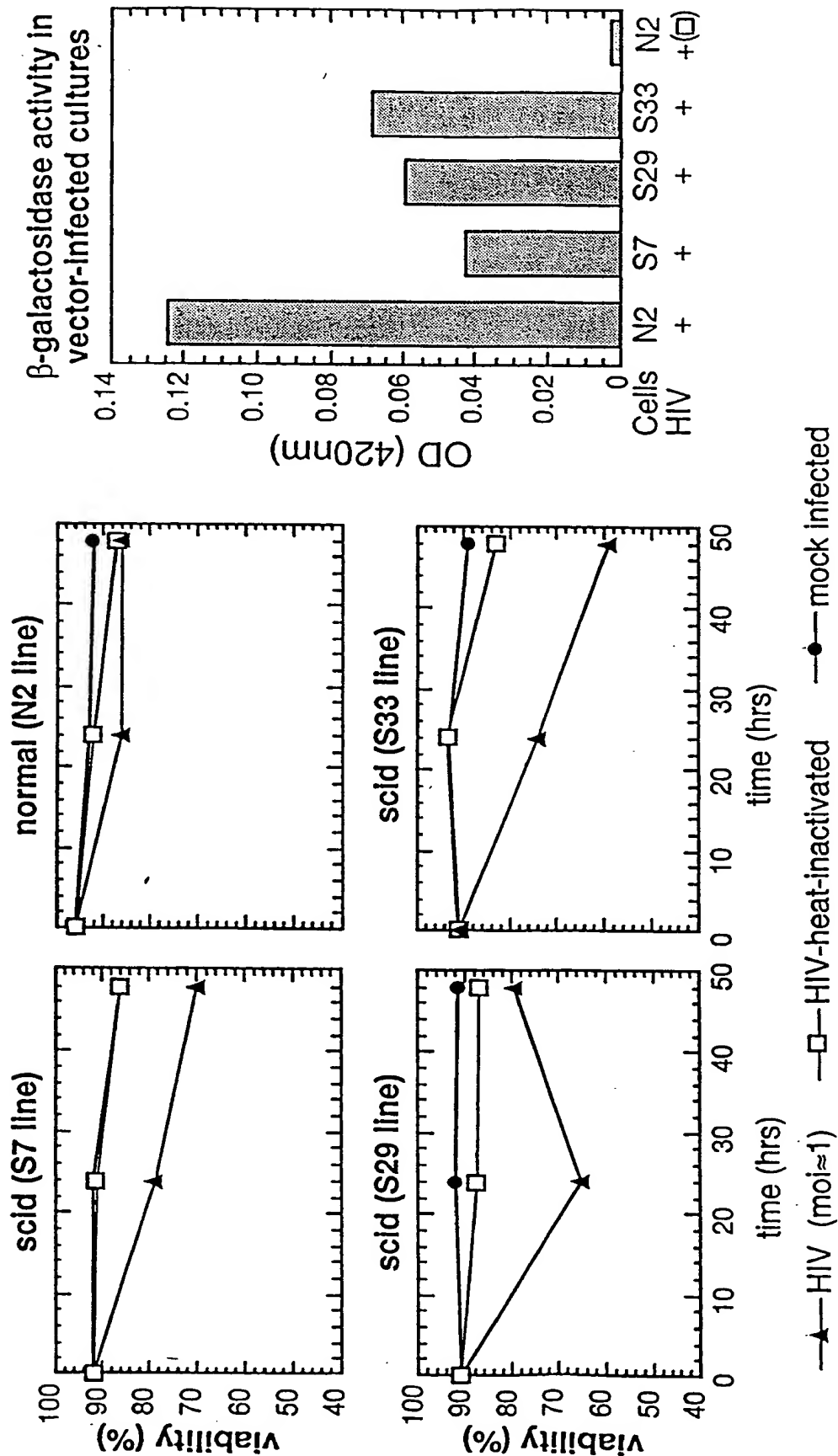


Figure 6

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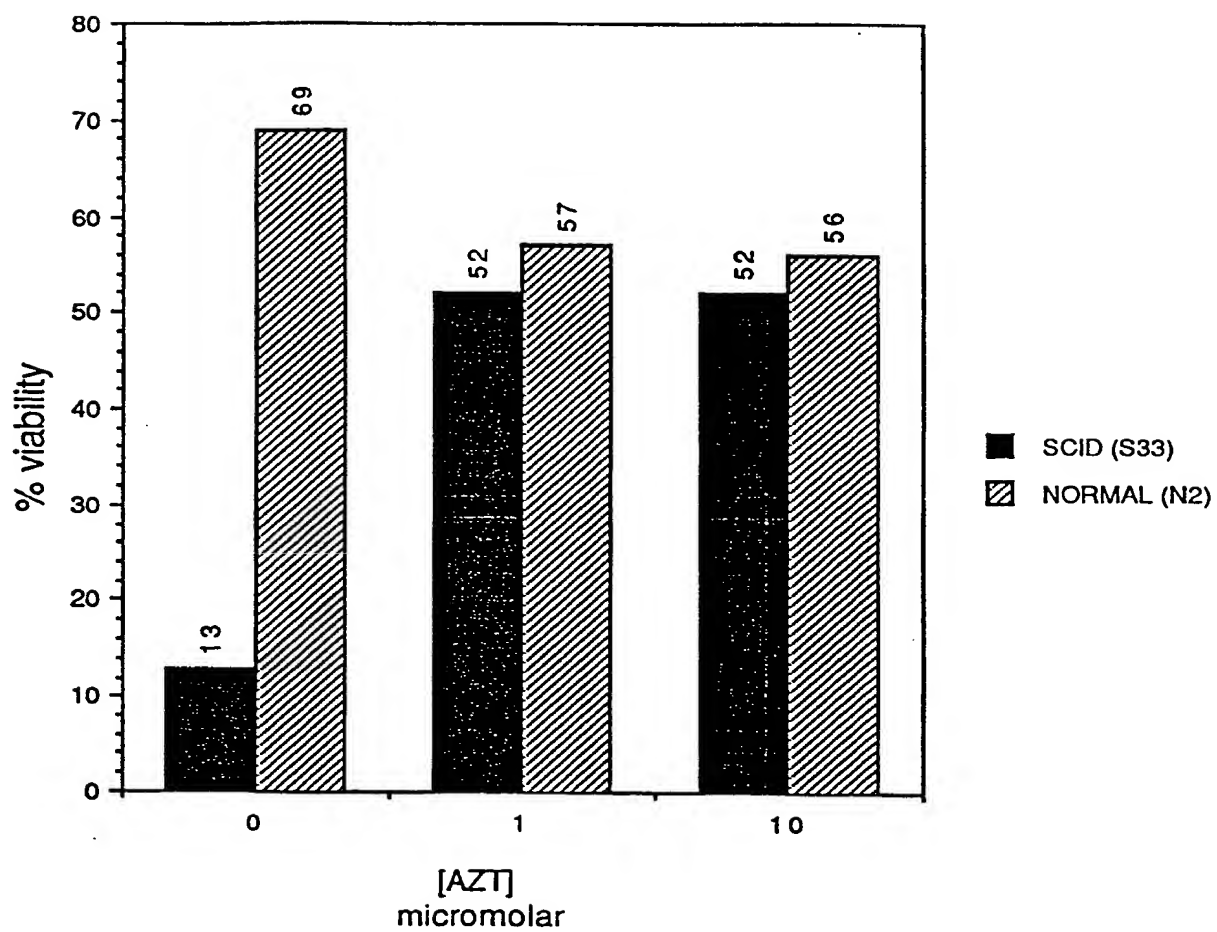


Figure 7

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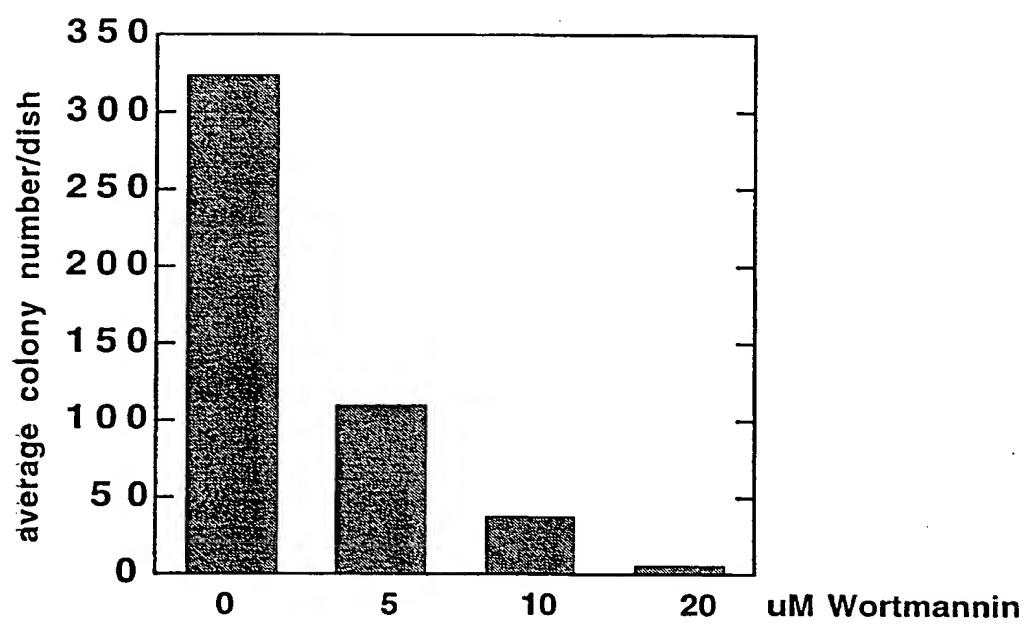


Figure 8

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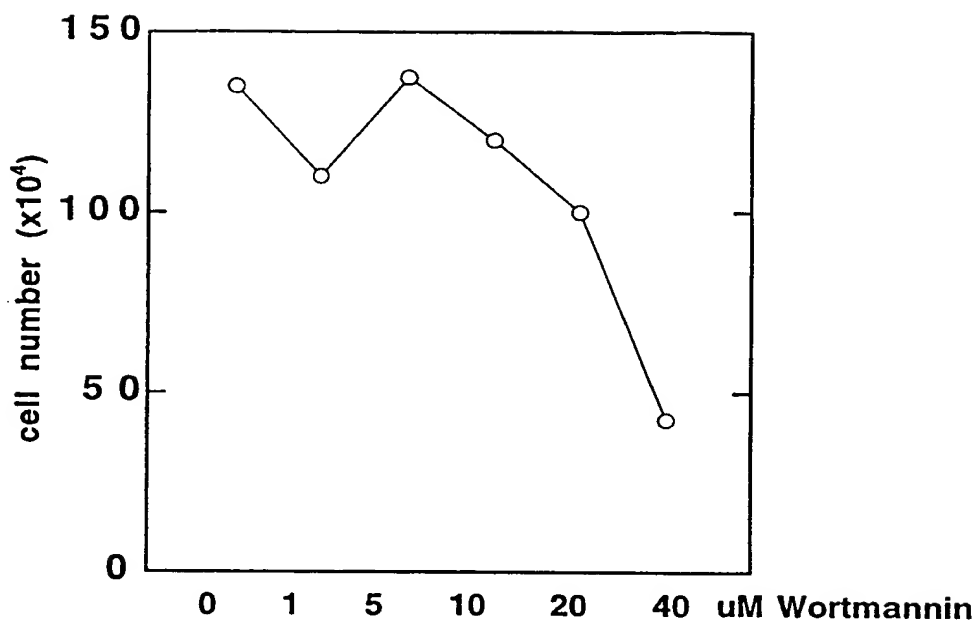


Figure 9

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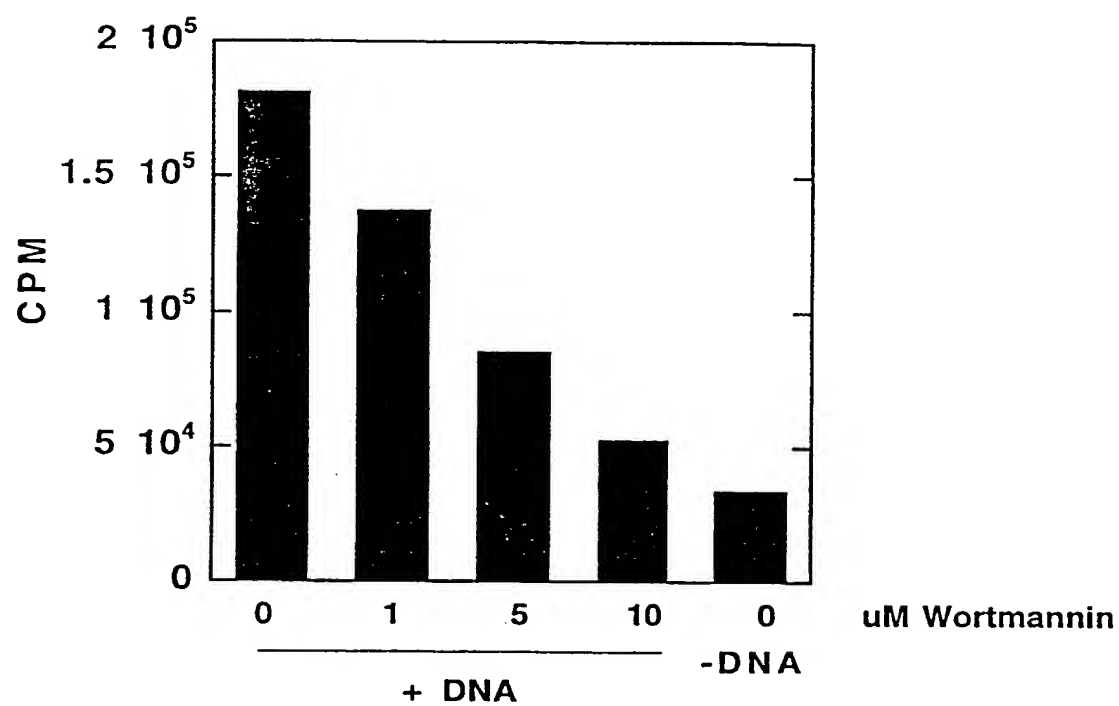


Figure 10

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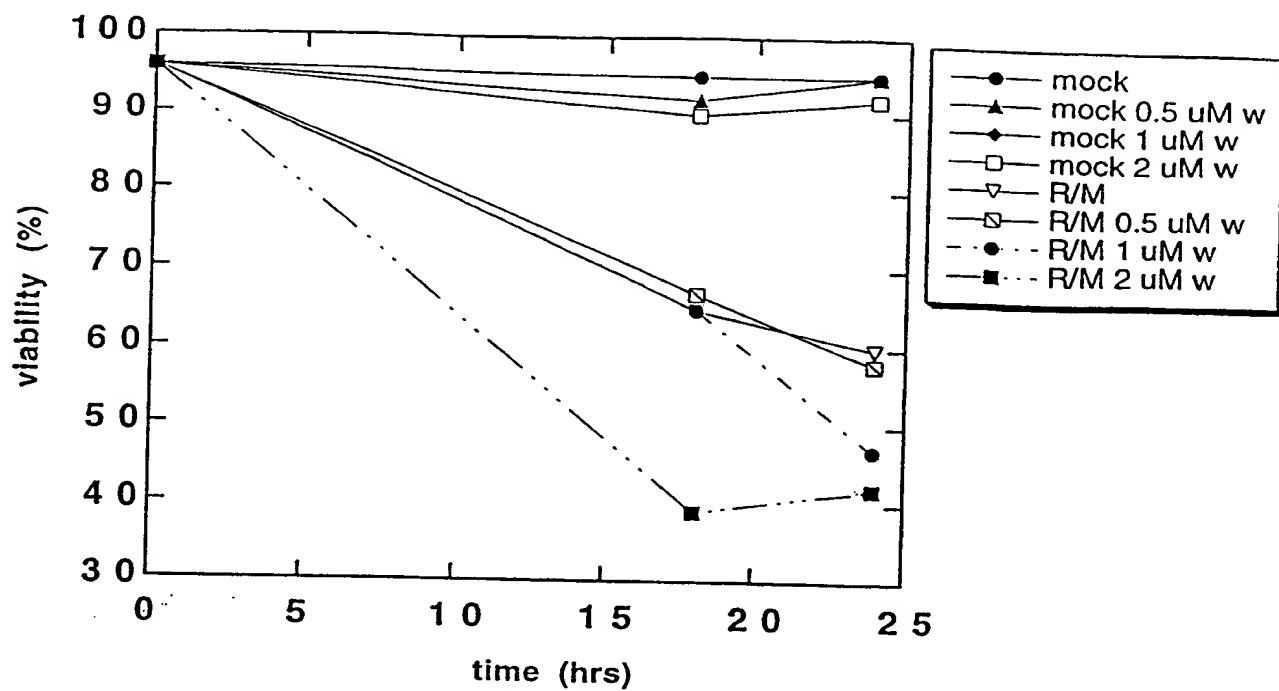


Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/21863**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12Q 1/00, 1/68, 1/70; C12N 13/00, 9/00; GO1N 33/573

US CL :435/4, 5, 6, 7.4, 173.3, 183

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 5, 6, 7.4, 173.3, 183

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, BIOSIS, USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	US 5,955,644 A (HASTY et al) 21 September 1999, see entire document.	1-34N
A,P	DANIEL, R., et al. A Role for DNA-PK in Retroviral DNA Integration, Science, 23 April 1999. Vol. 284, No. 5414, pages 644-647, see entire document.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 FEBRUARY 2000

Date of mailing of the international search report

02 MAR 2000

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